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Award Number: W81XWH-05-1-0073

TITLE: Identification of Novel Retinoid Targets in Prostate Cancer

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REPORT DATE: November 2006

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	**2. REPORT TYPE**	**3. DATES COVERED** *(From - To)*
01/11/06	Annual	1 Nov 05 – 31 Oct 06

4. TITLE AND SUBTITLE
Identification of Novel Retinoid Targets in Prostate Cancer

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-05-1-0073

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Sidney Kimmel Cancer Center
San Diego, CA 92121-1131

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT Retinoids have shown promise for the chemoprevention and treatment of prostate cancer. However, except for the efficient treatment of acute promyelocytic leukemia and certain skin disorders, most natural and synthetic retinoids have failed in clinical trials because of toxicity and limited activity. Retinoids exert their biological activity mainly upon binding and activation of the nuclear retinoid receptors (RARs and RXRs). Novel synthetic retinoid-related molecules (RRMs) that show selective activity towards RARã/â (MX3350-1, CD2325) or function as RAR antagonists (MX781) have been discovered that elicit strong anticancer activity and represent promising leads for the chemoprevention and treatment of prostate cancer. These RRMs induce apoptosis independently of RARs; instead, they bind to other cellular proteins to elicit profound effects on the cell signaling events that lead to inhibition of cell growth and induction of programmed cell death. The cellular targets that mediate RRM-anticancer activity are unknown and the molecular mechanism of RRM action is currently under extensive investigation. Our goals were to identify genes that mediate RRM anticancer activity upon selection of Genetic Suppressor Elements (GSE) that confer resistance to RRM treatment in prostate cancer cells. GSE expression is expected to inhibit RRM-induced apoptosis by blocking the function of key genes that are critical for the anticancer activity of RRMs. After standardizing the experimental conditions to achieve optimal retrovirus production and infection of PC3 cells, we have performed several screenings in the presence of toxic amounts of MX781 and MX3350-1. GSEs have been subsequently rescued from surviving cells by PCR amplification using primers specific for the GSE library, followed by identification by DNA sequencing. Several genes, many of them with biological functions unrelated to apoptosis, have been identified as potential candidates of interest for functional validation. Of particular interest are certain protein kinases with a role in apoptosis, and proteins that play a role in mitochondrial function and/or oxidative stress.

15. SUBJECT TERMS
Retinoids; Genetic Suppressor Elements; Target Identification

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON**
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**			USAMRMC
U	U	U	UU	8	**19b. TELEPHONE NUMBER** *(include area code)*

Standard Form 298 (Rev. 8-98)
Prescribed by ANSI Std. Z39.18

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Introduction.

Retinoids are natural and synthetic derivatives of vitamin A that bind and activate the nuclear retinoid receptors (RARs and RXRs) to regulate the expression of target genes. Because of their differentiation and growth inhibitory activities, many efforts have been devoted to develop retinoids as cancer preventive and chemotherapeutic agents. However, very few compounds are clinically useful to humans beyond the treatment of APL and dermatological disorders. This is mostly because of the high levels of toxicity observed at effective retinoid doses, which are most probably caused by activation of the retinoid receptors. Synthetic derivatives have been obtained that selectively activate a subset of retinoid receptors or lack of transactivation activity, which are expected to show lower toxicity. Of particular interest to our program are the adamantyl containing retinoid-related molecules (RRMs) MX3350-1 and MX781. These compounds induce apoptosis in a variety of cancer cell lines and MX3350-1 is effective in animal models against solid tumors derived from non-small cell lung carcinomas, whereas MX781 is effective in breast cancer xenograft models. These molecules are strong inducers of apoptosis in prostate carcinoma cells independently of p53 status, and therefore represent promising leads for the discovery of novel retinoid-like molecules as chemopreventive agents in prostate cancer.

Body.

The main goal of our research program is to understand the mechanism of RRM action and to identify genes that mediate their anticancer activity in prostate carcinoma cells. The discovery of genes that are implicated in RRM killing could have a tremendous impact in: i) understanding the mechanism of RRM action in prostate cancer and ii) the discovery of novel therapies that might synergize with currently available retinoids in combination therapies. For this purpose we proposed a Genetic Suppressor Elements (GSE) selection approach as a way to identify genes that mediate RRM action in prostate cancer. GSEs are small fragments of DNA produced by random digestion of a cDNA library that function as antisense DNA (when oriented in antisense direction and are able to decrease expression of a target gene) or as dominant negative fragments of a particular gene product (when expressed in sense orientation). GSEs derived from genes that are required for RRM-induced apoptosis are expected to block cell death in response to RRM treatment. We hypothesized that transfection of a GSE library into PC3 cells should generate cellular clones that would be resistant to RRM killing. GSEs isolated from cells surviving a killing dose of RRM would help us identify genes that mediate RRM function.

The Pantropic Retroviral Expression system (BD Biosciences, K1063-1) has been utilized to generate retroviruses carrying a GSE library, which have subsequently been used to infect PC3 cells prior to RRM treatment and GSE selection. During the first year of the project we standardized transfection conditions for optimal virus production and infection of PC3 cells. A first large-scale experiment was initiated and cells resistant to MX3350-1 were isolated for subsequent GSE rescue. Genomic DNA was prepared using a DNeasy Tissue kit (Qiagen) and 0.5 µg DNA was used as a template for PCR amplification of GSEs using the following primers: GAGAGCAGATCTACGCGTCGACATGGTAT (forward) and CAGTCAG ATCTGCGGTCGACTAGTCA (reverse), which correspond to the adapters used to create the GSE library and clone into the pLmGCX expression vector. GSE amplification was performed with KlenTaq LA DNA polymerase (Sigma), which is a mixture of KlenTaq-1 DNA polymerase and a small amount of a proofreading DNA polymerase. This enzyme blend exhibits increased fidelity and yield of the DNA product and is ideal for amplifications of small DNA fragments from genomic templates. PCR conditions were standardized as to observe optimal amounts of DNA. As positive control for PCR amplification, we used the original GSE library as a template (see Fig. 1 below). The PCR product was digested with Bgl II, purified by agarose gel, extracted from the gel using a DNA gel extraction kit (Qiagen), and ligated with Bgl II-linearized pLmGCX vector. Four microliters of

ligation mixture were used to transform *E. coli* DH5α competent cells. A control ligation/transformation experiment was performed in parallel with linearized vector alone, which yielded a very low number of Amp resistant colonies as opposed to the GSE/pLmGCX mix (not shown). Plasmid DNA was prepared following standard procedures for a second round of GSE selection.

Following identical conditions as with the first selection, we transfected $10x10^6$ GP2-293 cells per plate (10 plates 150 mm) with 10 µg of library DNA containing the enriched GSEs (or empty pLmGCX vector in one 150 mm plate) and 5 µg of pVSV-G. 20 hours after transfection, the medium containing the DNA precipitates was removed, cells were washed with PBS, and fresh medium was added. At this time, PC3 cells were seeded in eleven 150 mm dishes to carry out the viral infection. 24, 48, and 72 hours post transfection, the supernatant from the GP2-293 containing dishes was collected, filtered through Millex-HA 0.45 µm filter units (Millipore), and added onto the PC3 plates. Virus supernatant was left for 6 hours, when medium was replaced by fresh DMEM medium containing 10% FBS. 24 hours after the last infection, cells were treated with 6 µM MX3350-1 in medium containing 1% serum for 48 hours, enough to kill 100% of noninfected PC3 cells. Subsequently, cells were grown in the presence of 10% serum for 2 additional weeks. Surviving cells were pooled and genomic DNA was prepared for subsequent GSE rescue. Using similar amplification conditions as described above, we rescued the GSEs from the surviving cell population. The PCR product was cloned into a pCR II blunt expression vector (Invitrogen) and 100 individual colonies were grown for DNA sequencing. A BLAST homology search indicated that all DNA sequences with the exception of two belonged to different genes; only two sequences were repeated, which matched to the glutathione peroxidase-1 (GPX1) gene (accession # AY327818) (data not shown). True GSEs should appear a repeated number of times in the sequenced colonies. More reliable results are obtained when one gene is represented by two or more different GSEs. Therefore, the results obtained in these first screenings after two rounds of selection were not very encouraging.

One potential explanation for this negative result is that synthetic RRMs activate different pathways to induce apoptosis, which may function independently of each other or cooperatively to accelerate the process of apoptosis. Thus, expression of one or more GSEs blocking one particular pathway might not necessarily prevent RRM-induced apoptosis from occurring via a secondary alternative pathway. A second explanation is that certain GSEs are not very strong or their expression in the target cells is limited to a short period of time. To circumvent these problems, we re-designed the GSE selection strategy and decided to harvest surviving PC3 cells early after a short RRM treatment (24 to 48 hours). Although some of the control pLmGCX-transduced PC3 cells are still alive after 48 hours, we expected that a significantly larger number of cells would survive when infected with viruses carrying the GSE library. We reasoned that if several pathways are activated by RRM that converge in cell death, blocking one pathway would delay the whole process of apoptosis. Furthermore, by collecting cells after a short period of RRM treatment we would avoid loosing weak GSEs and/or GSEs expressed for short periods of time. To obtain meaningful data, early recovery of GSEs will require a high-throughput sequencing of several hundreds of colonies to look for sequence enrichment.

We scaled-up virus production by transfecting GP2-293 cells in a CellSTACK culture chamber (Corning) containing 10 stacks with 6,360 cm^2 cell growth area (equivalent to 42 of the 150 mm culture dishes, enough for two to three drug screenings). 320 million GP2-293 cells were incubated with a mixture of Calcium Phosphate/BBS containing 2 mg pLmGCX-GSE library and 1 mg pVSV-G vector. The cell/DNA mixture was added into a poly-D-Lysine treated CellSTACK, placed inside a 3% CO_2 incubator, and incubated for 16 hours. An aliquot of the cell/DNA mix was added into a p100 dish to follow up under the microscope and a control transfection was carried out in parallel with empty pLmGCX vector. After transfection, the medium was carefully removed, cells were washed with PBS, and fresh medium was added; supernatant containing virus was collected each day for the following 5

days, filtered, and quickly frozen at -80 °C. One aliquot for each day stock was reserved for virus titration.

For GSE selection, we seeded PC3 cells into 15x150 mm culture dishes per drug treatment. As indicated before, we infected three times in 24 hour intervals. Twenty-four hours post-infection, cells were treated with 6 µM MX3350-1 or 6 µM MX781. PC3 cells were harvested after 48 hours of RRM treatment. Genomic DNA was isolated using a DNeasy Tissue kit (Qiagen) and analyzed by agarose gel electrophoresis. PCR amplification was subsequently performed using 0.5 µg genomic DNA as template and 2.5 U of KlenTaq LA DNA polymerase. As negative control, genomic DNA isolated from non-infected PC3 cells was used. In addition, 0.1 µg of pLmGCX-GSE DNA was used as positive control (Fig 1). The PCR products were cloned into the pCR II-expression vector using a Zero Blunt TOPO PCR cloning kit (Invitrogen). Several hundreds of colonies were obtained from each drug screening.

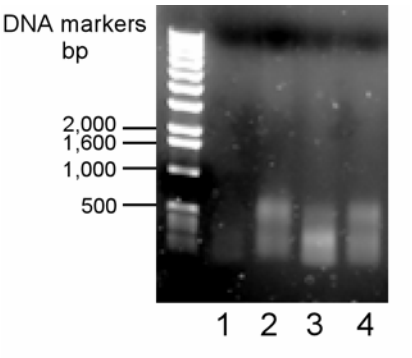

Figure 1. 1% agarose gel analysis of PCR products obtained using genomic DNA from non-infected PC3 cells (lane 1), pLmGCX-GSE library (lane 2), or genomic DNA from GSE-infected PC3 cells treated with MX781 (lane 3) or MX3350-1 (lane 4). Note that the PCR products represent a mixed population of DNA fragments ranging in size between 100 and 500 bp. As expected, DNA isolated from non-infected cells gave no detectable PCR product using primers specific for the creation of the GSE library.

Individual colonies from the MX781-screening were grown in 1.5 ml LB medium for DNA preparation. Positive colonies were identified following Bgl II digestion (present in both primers/adapters flanking the GSE sequence, but not in the pCR II vector; see figure 2), arrayed in 8x96 plates, and submitted for DNA sequencing. To identify the corresponding genes, DNA sequences were analyzed by BLAST homology searches in the NCBI databases. Table I shows the genes that were represented in two or more clones, which could indicate targets of interest for functional validation. As expected, we found both sense and antisense fragments of the same gene.

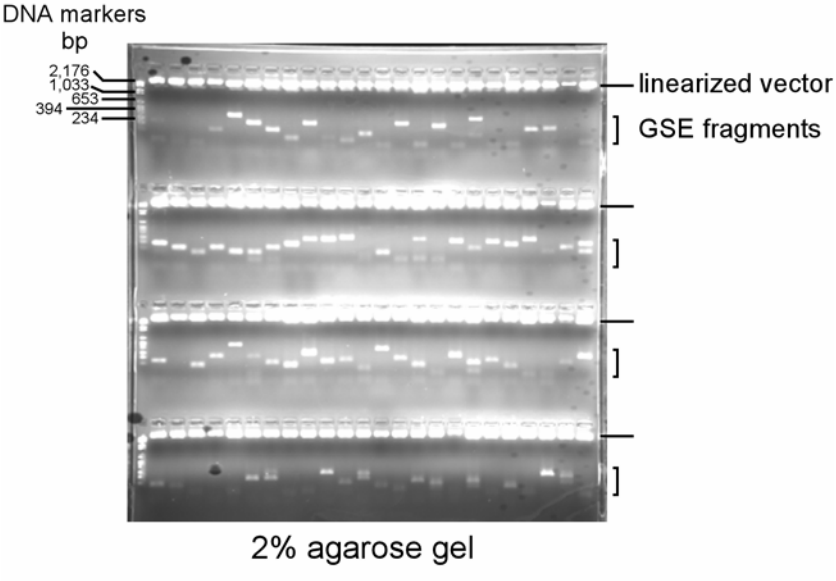

Figure 2. Agarose gel analysis of Bgl II-digested DNA from 96 colonies. To estimate the size of the GSE insert, we used a 2% agarose concentration. About 80% of the colonies show DNA inserts of 100 to 500 bp in length. Some colonies were empty whereas a limited number of them had two clearly identifiable inserts, which could be due to insertion of two DNA fragments during library construction. DNAs with a defined fragment of ≥100 bp were considered positive and sequenced.

Table I. Genes represented by two or more GSEs isolated from the MX781 screening. The number of sequences that appear in sense (s) or antisense (as) orientation is shown.

	Gene	Sequences (s/as)	Accession Number
ADAM17	ADAM metallopeptidase with thrombospondin type 1 motif 17	4 (as)	NM_003183
ANCO	Ankyrin repeat and SOCS box-containig	1 (s), 3 (as)	AY533563
ARG2	Arginase type 2 mitochondrial protein	5 (s)	NM_001172
DHX35	DEAH box polypeptide 35 (ATP-dependent helicase)	3 (as)	NM_021931.
CSK	c-src tyrosine kinase	6 (s)	NM_004383.
CYP27A1	Cytochrome P450, family 27, subfalily A (Vit D3 25-hydroxylase)	4 (s)	NM_000784
DCTN	Dynactin 2	2 (s)	NM_006400
EXOC7	Exocyst complex component 7	3 (s)	NM_001013839
FALZ	Fetal Alzheimer antigen	1 (s), 1 (as)	NM_004459
FLNA	Filamin A (actin binding protein)	4 (as)	NM_001456
GEF	Rho guanine nucleotide exchange factor	9 (s)	NM_015313.
GPX1	Glutathione peroxidase 1	2 (s), 3 (as)	NM_000581
HR	Hairless homolog (mouse)	3 (as)	NM_018411
ICMT	Isoprenylcysteine carboxyl methyltransferase	1 (s), 2 (as)	NM_012405
LIMS3	LIM and senescent cell antigen-like domain	2 (s), 2 (as)	NM_033514
NPIP	Nuclear pore complex interacting protein	5 (as)	NP_008916
NOMO2	NODAL modulator 2	2 (s)	NM_001004060
NUP188	Nucleoporin 188 kDa	3 (as)	BC111045
PPIH	Cyclophilin H, peptidylprolyl isomerase H	4 (as)	NM_006347
PQBP1	Polyglutamine binding protein	5 (s)	NM_001032385
PRDX2	Peroxiredoxin 2	4 (as)	NM_181738
RPS23	Ribosomal protein S23	2 (as)	NM_001025.
SPRY2	Sprouty-related 2 (Drosophila)	3 (as)	NM_005842
STMN1	Stathmin 1	2 (as)	NM_203401
THAP7	Thanatos-associated protein 7	4 (as)	NM_001008696
TUBB2C	Tubulin beta 2C	2 (as)	NM_006088
UBL7	Ubiquitin-like 7	5 (s)	NM_201265
WDR 34	WD repeat domain 34	6 (as)	NM_052844
YWHAZ	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein	2 (as)	NM_003405.

None of the genes depicted in Table I have an obvious role on apoptosis, although some are protein kinases and important cell signaling molecules that might be required for MX781-induced cell death. GEF12 is the most frequently found gene; this is a Rho guanine nucleotide exchange factor; whereas certain GEFs function as oncogenes and promote cell proliferation, some GEF family members have been involved in apoptosis, including GEF-H1 and p115-RhoGEF. The fact that this sequence has been found with relatively high frequency in this screening encourages further validation assays with this gene. Although Csk has not been directly linked to apoptosis, the fly homolog dCsk has been shown to negatively regulate organ growth and proliferation by inhibiting c-Src, JNK, and STAT pathways. Csk, as well as c-Src have also been shown to be activated by oxidative stress, which plays an important role in MX781-induced apoptosis (our unpublished observations). Therefore, Csk is an interesting target for functional validation. Other GSEs represent genes that function in cell signaling and cell cycle progression, including NPIP (nuclear pore complex interacting protein), the human homolog of SPRY2 (Drosophila Sprouty-related 2), and WDR 34 (WD repeat domain 34). Since our preliminary results show that MX781 induce apoptosis via mitochondrial damage and oxidative stress, GSEs corresponding to proteins with a role in mitochondrial function and the redox state of the cell, such as ARG2 (arginase type 2), GPX-1 (glutathione peroxidase 1), PRDX2 (peroxiredoxine 2), are of particular significance for

subsequent functional studies. Interestingly, GPX1 was the only gene that was found twice in our original screening with MX3350-1. This RRM also induces oxidative stress in prostate carcinoma cells (data not shown) and therefore may share with MX781 this oxidative pathway. Although GPX-1 is an antioxidant enzyme and prevents oxidative stress-induced apoptosis, it is possible that drug treatment converts GPX-1 into a pro-apoptotic molecule in prostate carcinoma cells. This warrants future validation of GPX-1.

We are currently analyzing the DNA sequences obtained from the MX3350-1 screening and results will be provided in the following report.

Although all the genes that appear two or more times in the rescued GSEs are good candidates for validation, we have repeated the screening with both drugs and we will sequence additional colonies to evaluate the reproducibility of these results and narrow down the number of candidate genes that will be validated by functional studies.

Research accomplishments.

- Standardization of large-scale transfection of GP2-293 cells and virus production in a CellStack Culture Chamber.
- GSE screenings with cytotoxic concentrations of MX3350-1 and MX781.
- GSE rescue and identification by PCR and high-throughput DNA sequencing.
- Prioritarization of GSEs conferring resistance to MX781 treatment.
- Identification of ARG2, GPX1, PRDX2, CSK, GEF12, WDR 34, as potential genes of interest for functional validation.

Reportable Outcomes.

No reportable outcomes are available at this time.

Conclusions.

During the second year of the project we have performed several screenings with two RRMs, the agonist MX3350-1 and the RAR antagonist MX781. Although initial screening did not produce any useful data, subsequent changes in the GSE selection strategies yielded significant results that are currently being validated. Because of the risky nature of this project, these changes in the research strategy are sometime necessary in order to obtain meaningful data. The project is progressing as expected and we expect to finalize validation of the rescued GSEs during the third and last year with no further delays.

References. None.

Appendices. None.